

GAS CHROMATOGRAPHY AND MASS SPECTROMETRY
IN DIAGNOSTIC MICROBIOLOGY:
ANALYSIS OF VOLATILE BACTERIAL PRODUCTS
AND MYCOBACTERIAL CELLULAR CONSTITUENTS

by

Lennart Larsson

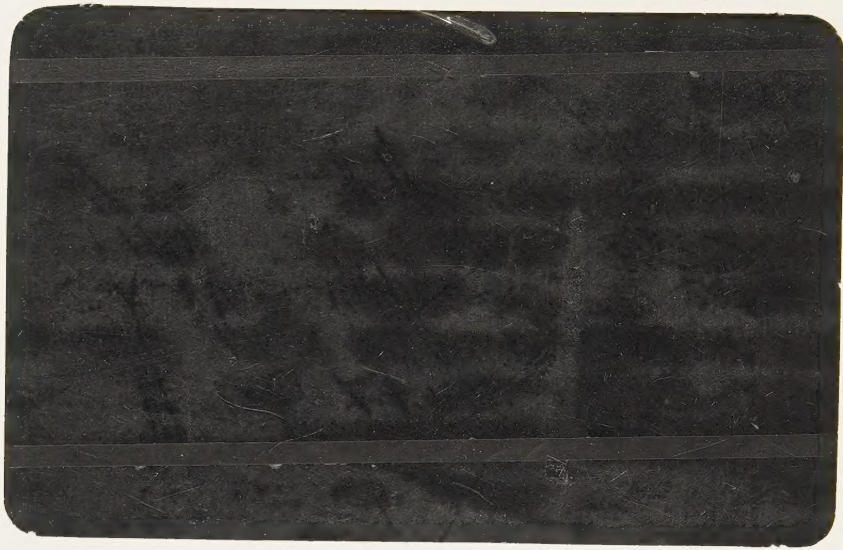
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Gas Chromatography and Mass Spectrometry in Diagnostic Microbiology: Analysis of Volatile Bacterial Products and Mycobacterial Cellular Constituents.

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Gas chromatography and mass spectrometry were used for the registration and identification of bacterial products. Mycobacterial carbohydrate and lipid constituents were analysed as trifluoroacetyl derivatized methyl glycosides and fatty acid methyl esters, respectively. Each of the mycobacterial strains studied could be characterized by its chromatographic pattern.

A gas chromatographic head-space technique for the analysis of volatile bacterial products was developed. The technique allowed registration of alkaline, neutral and acidic compounds produced by aerobic and strict and facultatively anaerobic bacteria. The method provided a high sensitivity in detecting short-chain alcohols and amines.

Selected ion monitoring was applied for the detection of tuberculostearic acid in mycobacteria and nocardia. This fatty acid could be detected in mycobacterial cultures before colonies could be visually observed. It was also demonstrated in sputum specimens from patients with pulmonary tuberculosis.

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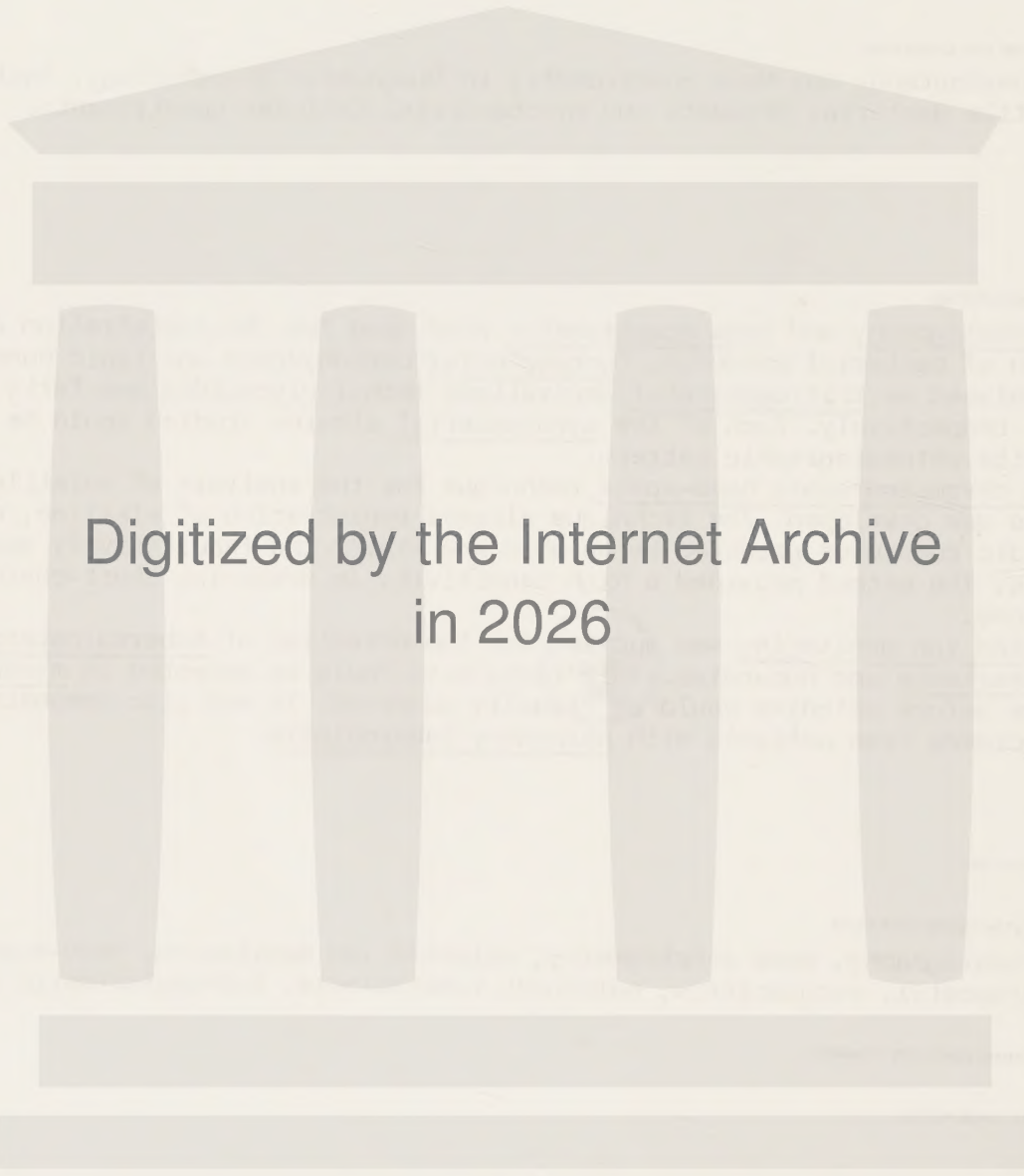
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GAS CHROMATOGRAPHY AND MASS SPECTROMETRY IN DIAGNOSTIC MICRO-
BIOLOGY: ANALYSIS OF VOLATILE BACTERIAL PRODUCTS AND MYCO-
BACTERIAL CELLULAR CONSTITUENTS

This review summarizes and discusses results from papers
I-VI:

- I. L. Larsson, and P.-A. Mårdh. Gas Chromatographic Characterization of Mycobacteria: Analysis of Fatty Acids and Trifluoroacetylated Whole-Cell Methanolysates. J. Clin. Microbiol. 3 (1976) 81.
- II. L. Larsson, R. Bergman, and P.-A. Mårdh. Gas Chromatographic Characterization of Porcine and Human Strains Belonging to the *Mycobacterium avium-intracellulare* Complex. Acta path. microbiol. scand. Sect. B. In press.
- III. L. Larsson, P.-A. Mårdh, and G. Odham. Detection of Alcohols and Volatile Fatty Acids by Head-Space Gas Chromatography in Identification of Anaerobic Bacteria. J. Clin. Microbiol. 7 (1978) 23.
- IV. L. Larsson, P.-A. Mårdh, and G. Odham. Analysis of Amines and Other Bacterial Products by Head-Space Gas Chromatography. Acta path. microbiol. scand. Sect. B 86 (1978) 207.
- V. L. Larsson, P.-A. Mårdh, and G. Odham. Detection of Tuberculo-stearic Acid in Mycobacteria and Nocardiae by Gas Chromatography and Mass Spectrometry Using Selected Ion Monitoring. J. Chromatogr. Biomed. Appl. In press.
- VI. G. Odham, L. Larsson, and P.-A. Mårdh. Demonstration of Tuberculo-stearic Acid in Sputum from Patients with Pulmonary Tuberculosis by Selected Ion Monitoring. J. Clin. Invest. In press.

INTRODUCTION

In recent years, instrumental analytical techniques have been increasingly applied in diagnostic microbiology. With the aid of such techniques, microorganisms can often be detected and identified more rapidly, and with greater certainty, than when using traditional diagnostic methods such as biochemical tests, morphological studies etc. Physical and chemical characteristics of microorganisms can be determined either by means of instruments developed for general analytical purposes, or by using apparatus specially designed for the analysis of microorganisms.

Gas chromatography (GC) is a well established technique for both qualitative and quantitative analysis of volatile organic and inorganic compounds. In microbiology, GC has been used to determine microbial products, thus making it possible to characterize an organism by its chromatographic pattern. Microorganisms can often be identified by GC as to genus or even species.

Particularly when combined with GC, mass spectrometry (MS) is a method with a high potential for the elucidation of chemical structures, for example those of microbial metabolites. In addition, the adaptation of GC/MS for "selected ion monitoring" (SIM) allows the mass spectrometer to be used as a highly sensitive and selective GC detector for quantitative determination of such products. Since microorganisms can produce compounds characteristic of a given genus or species, SIM constitutes a technique with a wide scope of interest within clinical microbiology.

The following abbreviations have been used:

GC = Gas chromatography

HSGC = Head-space gas chromatography

MS = Mass spectrometry

m/z = Mass-to-charge ratio

SIM = Selected ion monitoring

TFA = Trifluoroacetyl

TMS = Trimethylsilyl

The work presented here deals with different GC and MS techniques, employed for the detection and diagnosis of bacteria. The prospects of adapting these methods for use in microbiological diagnostic laboratories are considered.

CONCEPTS OF GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

Gas chromatography

Dating back to 1952 (1), this technique is today an extensively employed separation method for both analytical and preparative purposes. The sample is introduced into an injection port (or directly onto the GC column), the temperature of which should be kept high enough to vaporize each sample component instantaneously. The plug of vapour so obtained is then swept through the column by the carrier gas stream. Separation of the sample components arises from differences in their interactions with the stationary phase in the column. Provided that the detector responds to each individual component, these will be registered as a series of peaks (the chromatogram) on a recorder. The area under each peak is related to the amount of the corresponding compound. In addition, under constant chromatographic conditions, the retention time of a given compound is defined and can be calculated from the chromatogram (2,3).

GC thus allows qualitative and quantitative determinations of compounds that are either volatile, convertible to volatile derivatives, or degradable to volatile products. Derivatization of non-volatile compounds, and compounds having high boiling points, usually involves removal of one or several active hydrogen atoms in the molecule. Polymers generally require chemical or thermal degradation to corresponding monomers before being chromatographable. Recent development in GC particularly concerns the use of selective detectors and high resolution columns, allowing the separation of complex samples of, *inter alia*, biological origin.

Mass spectrometry

This method is one of the most useful techniques available for the elucidation of molecular structures of organic compounds

4.
(3,4). In Fig. 1, an electron impact magnetic single-focusing mass spectrometer is shown schematically.

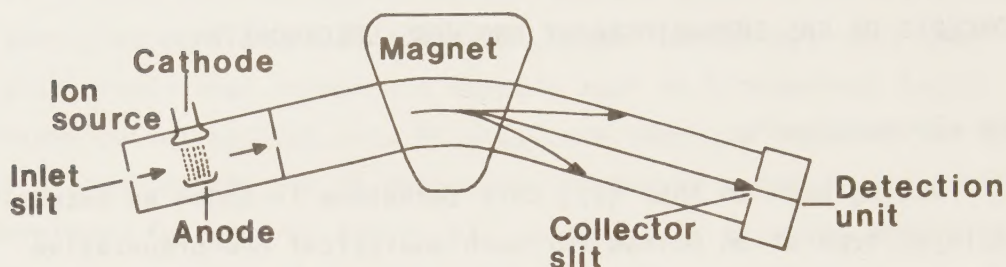


Fig. 1. Schematic diagram of an electron impact magnetic sector mass spectrometer.

Sample molecules are bombarded with electrons in the ion source in order to form a variety of products, including positively charged ions. These are then accelerated through the analyser tube by means of an electrostatic field. At a defined acceleration voltage and magnetic field strength, exclusively ions of a specific mass-to-charge ratio (m/z) will reach the collector slit and enter the detection unit. To obtain a complete spectrum, either the magnetic field strength or the acceleration voltage can be varied continuously. Thus, ions of different m/z values will be registered successively and a mass spectrum will result.

Chemical ionization provides an alternative to the electron impact method. Ions formed by electron bombardment of methane or other gases are used for further reaction with the sample molecules. Mass spectra obtained by chemical ionization often exhibit very intense peaks at m/z $M-1$ (hydrocarbons) or m/z $M+1$ (hetero-atom-containing molecules), where M indicates the molecular weight of the compound analysed (4).

Generally speaking, a given mass spectrum corresponds exclusively to one molecular structure. The peak of the highest m/z value often corresponds to the molecular ion, whose weight can consequently be directly determined. Other peaks in the mass spectrum represent different cleavage fragments. Identification of the compound studied can be accomplished by theoretical considerations or by simply comparing the mass spectrum obtained with those of known compounds.

Gas chromatography/Mass spectrometry (GC/MS). GC as an inlet system to the mass spectrometer offers several advantages. The sample components are separated in the column before entering the ion source. The specificity and sensitivity of MS combined with GC retention data makes GC/MS an extremely valuable analytical system.

Selected ion monitoring (SIM) implies the use of pre-selected acceleration voltages and magnetic field strengths throughout the entire analysis. The mass spectrometer will consequently monitor at a limited number of ions, resulting in a considerably improved sensitivity. This is primarily due to the fact that the output from the electron multiplier can be integrated over a longer time interval, thus enhancing the signal-to-noise ratio. The ions selected for monitoring should be of a high intensity and possess a relative specificity for the compound studied. The selectivity gains from monitoring at two or more mass numbers (multiple ion monitoring), though the sensitivity is thereby weakened, compared with that obtained when using single ion monitoring (4,5).

ANALYSIS OF MICROORGANISMS BY GAS CHROMATOGRAPHY OR GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Since 1963 when it was first suggested that GC could be used to identify microorganisms (6,7), a steadily increasing number of reports dealing with this application of GC have been published (*c.f.* ref. 3). Microbial cellular compounds may be chromatographed after being degraded and possibly derivatized. Furthermore, extra-cellular microbial products can be studied by GC and GC/MS. Table 1 shows some of the microorganisms, and Table 2 their metabolites,

Table 1. Some microorganisms that have been analysed by gas chromatography or gas chromatography/mass spectrometry.

Genus	References
<i>Achromobacter</i>	26,33
<i>Aerobacter</i>	6,64,66,71,73
<i>Bacillus</i>	6,14,15,36,37,41,64,66
<i>Bacteroides</i>	10,54,65,72
<i>Bifidobacterium</i>	58,65,73
<i>Clostridium</i>	8,28,30,54,56,57,59,65
<i>Corynebacterium</i>	65,73
<i>Escherichia</i>	6,21,41,55,60,62,63,64,66,68,71,73
<i>Klebsiella</i>	6,21,60,62,63,73
<i>Lactobacillus</i>	58,65,66,73
<i>Listeria</i>	46
<i>Micrococcus</i>	6,15,31,32
<i>Moraxella</i>	22,33,34,60
<i>Mycobacterium</i>	17-19,40,45,47,48,50,51
<i>Neisseria</i>	13,22-24,33-35,39,53,67
<i>Peptococcus</i>	52
<i>Peptostreptococcus</i>	52
<i>Propionibacterium</i>	54,65,72,73
<i>Proteus</i>	6,55,61-63,66
<i>Pseudomonas</i>	25,42,43,49,60,64,68,69,71,72
<i>Salmonella</i>	60,66,68,71
<i>Serratia</i>	6,60,62,63,66
<i>Staphylococcus</i>	21,32,62,63,66,72
<i>Streptococcus</i>	11,12,20,21,27,41,62,63,66,68
<i>Treponema</i>	29,44
<i>Acholeplasma</i>	15
<i>Actinomyces</i>	65,73
<i>Aspergillus</i>	73
<i>Candida</i>	63
<i>Rhizopus</i>	15
<i>Saccharomyces</i>	15,41,73

Table 2. Some microbial products that have been analysed by gas chromatography or gas chromatography/mass spectrometry.

Compounds studied	References
<i>Cellular products</i>	
amino acids	44
carbohydrates	30,32,34,38,53
fatty acids	6,26,27,30-33,36-43,45-53
pyrolysis products	7,9-21
<i>Extracellular products</i>	
acetoin	64,66,71,73
alcohols	56,57,62-66,70,71,73
amines	55,56,60,67,68
diacetyl	64,70,71,73
fatty acids	23-26,43,54,56-59,64-66,69-73
sulphur-containing compounds	61,62,63

which have been analysed by GC or GC/MS. (For a more extensive review, see also L. Larsson and P.-A. Mårdh. *Acta path. microbiol. scand. Sect. B, Suppl. 259* (1977), 5-15 (8).)

Analysis of cellular products

Whole cells of microorganisms, and extracts of such cells, must be degraded to volatile fragments before being introduced to the GC column. This can be done either thermally or chemically. Thermal decomposition, *viz.* pyrolysis, implies heating pellets of (generally lyophilized) organisms to 400-1300°C (7,9-21). The pyrolysis products so obtained are then swept through the column by the carrier gas stream. The resulting chromatographic patterns, *viz.* pyrograms, are generally characteristic of each microbial species studied, provided that highly standardized test conditions are being used. In order to separate as many as possible of the

fragments obtained during a single pyrolysis, open tubular (capillary) columns are preferably employed (7,11-13,15,16,21). In addition, computers are normally necessary for interpretation of the often highly complex pyrographic patterns obtained (9,16,20,21).

Chemical decomposition usually involves hydrolysis, methanolysis or use of a digestive reagent, such as tetramethylammonium hydroxide (6,22,26-53). Methanolysis of lyophilized cells followed by derivatization using a trimethylsilyl (TMS) or trifluoroacetyl (TFA) reagent yield chromatograms mainly representing fatty acid methyl esters from lipids, and TMS or TFA derivatized methyl glycosides from carbohydrates (35,53). Chromatograms merely representing constituents of bacterial lipids, carbohydrates, or proteins can be obtained by selective extraction methods. Capillary columns have so far been only rarely employed. Evaluation of the GC data obtained has generally been accomplished through visual inspection of the chromatograms, although computers are increasingly used.

Analysis of extracellular products

Volatile metabolites, *e.g.* alcohols, amines, and fatty acids, are normally detected by GC analyses of broth cultures, or extracts thereof (54,58,64,65,66,71-73). Since direct injection of culture media may be deleterious to the GC column, chromatography of extracts of the media is commonly employed. Analysis by GC of volatiles present in the atmosphere above such media, *viz.* GC head-space analysis (59,61-63,70), can be used alternatively, also offering the advantage of being easily performed.

Non-volatile metabolites, and metabolites with high boiling points, must be extracted and converted to volatile derivatives before being chromatographed (23-26,43,54-58,60,65,67-69,72).

DIAGNOSIS OF INFECTIOUS DISEASES BY GAS CHROMATOGRAPHY OR GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Diagnosis of infectious diseases by analysis of body fluids represents a recent application of GC (55,74-84). To obtain a sensitivity high enough for detection of the metabolites studied,

derivatization by means of, *e.g.* halogen-containing reagents, and electron capture detection, can be used (55,75-77,79). Chromatographic patterns, characteristic of a given infectious disease, have been reported.

Thus, GC may offer a possibility to diagnose certain infectious diseases without the need to isolate the etiological agent(s). Detection by GC/MS of compounds, *unique* for a given infectious agent, constitutes a further advantage of such a diagnostic procedure (75).

AIMS OF THE PRESENT INVESTIGATIONS

The investigations were intended

- (a) to evaluate whether GC can be used as a tool in the differential diagnosis of mycobacteria by analysis of cellular carbohydrates and fatty acids (I,II);
- (b) to develop a GC head-space technique for the analysis of both acidic, neutral, and alkaline volatile bacterial products (III,IV);
- (c) to apply SIM for the selective registration of microbial products, *e.g.* tuberculostearic acid of mycobacteria and nocardiae (V);
- (d) to ascertain if SIM can be adapted for the rapid diagnosis of tuberculosis by detection of tuberculostearic acid in clinical specimens (VI).

GAS CHROMATOGRAPHIC CHARACTERIZATION OF MYCOBACTERIA (I,II)

Background

Tuberculosis is still one of the most important infectious diseases in the world (85). It is usually caused by *Mycobacterium tuberculosis*, although other mycobacterial species may (less often) be encountered, *viz.* *M. bovis* and "anonymous" or "atypical" mycobacteria such as *M. avium* and *M. kansasii*. These latter mycobacteria seem at present to be responsible for an increasing frequency of infections in man in developed countries (85). Many mycobacteria, such as *M. tuberculosis*, are slow-growing, why their diagnosis is time-consuming. In addition, the differential diagnosis of mycobacteria is a laborious process.

In order to facilitate the identification of mycobacteria, GC (among other techniques) has been applied. GC analyses of mycobacterial pyrolysis products (17-19), cellular fatty acids (40, 47,48,50,51), and products from the digestion of mycobacterial cells with methanolic tetramethylammonium hydroxide (45) have been carried out. The resulting chromatographic patterns have, at least in some instances, been considered typical for the mycobacterial strain or species studied.

Analysis of TFA derivatized methanolysates of whole bacterial cells by GC was described by Jantzen *et al.* in studies on *Neisseria* (35). Chromatograms obtained by this technique were reported to represent not only bacterial fatty acids but also carbohydrates. Furthermore, these chromatograms were more reproducible than those obtained when employing TMS derivatization. In the present study, it was therefore decided to estimate if the TFA technique could be used as a tool in the differential diagnosis of mycobacteria.

When lyophilized bacteria are treated with an excess amount of acidic (HCl) methanol, both free and linked fatty acids are converted into methyl esters in a few hours at 80°C. Simultaneously, carbohydrates - including polysaccharides - are fragmented and converted into methyl glycosides of monosaccharides (86-88). Other constituents of the bacteria, such as proteins, nucleic acids etc, are considered not to be hydrolysed under the prevailing reaction conditions (35).

TFA derivatization as an alternative to other means of volatilizing carbohydrates before being subjected to GC analysis, *e.g.* TMS derivatization and acetylation, offers several advantages. The TFA derivatives are heat-stable and volatile, meaning that the GC retention time is short when using a relatively non-polar GC stationary phase (86,88,89). What is more, trifluoroacetic anhydride, present in excess amounts in the sample to be analysed, is not deleterious to the flame ionization detector. Derivatization of monosaccharides and their methyl glycosides with trifluoroacetic anhydride has been studied extensively - complete reaction is obtained within a few minutes at 50-150°C (86,88,89).

Separation of fatty acid methyl esters by GC can be readily accomplished by using relatively non-polar methyl silicone stationary phases. These phases also possess a good thermal stability, why temperature programming can be used without any major disturbance of the base line. TFA derivatized methyl glycosides have been chromatographed using a variety of phases, including the above-mentioned (86). Thus the simultaneous analysis of derivatized fatty acids and carbohydrates does not constitute any particular difficulty.

Discussion of results

In study I chromatographic patterns resulting from analyses of mycobacterial cellular compounds using methanolysis were compared with those obtained when using methanolysis followed by TFA derivatization. The usefulness of the latter method in distinguishing mycobacteria belonging to the *M. avium-intracellulare* complex was also evaluated (11). As variations in the culture medium can affect the chemical composition of the mycobacterial cell wall (51), a chemically defined medium (Proskauer-Beck, Difco) was used. This medium had previously been reported to provide an acceptable growth rate for mycobacteria (47,48).

In Fig. 2, chromatograms obtained in analyses of *M. tuberculosis*, strain H₃₇R_v, are shown. The chromatographic pattern represents fatty acid methyl esters (A), while in (B) and (C) products obtained by the TFA derivatization technique are analysed. The most prominent GC peaks were identified by MS as TFA derivatized methyl glycosides and fatty acid methyl esters (unpublished results). The carbohydrate derivatives were detected in chromatograms (B) and (C) only. They were not satisfactorily separated when employing the packed column (B), and could be identified by MS only when using the capillary column (C) (Fig. 2).

Under the standardized preparative and analytical conditions used, the reproducibilities of the chromatograms obtained were striking. Analyses of different strains belonging to one and the same species resulted in virtually identical chromatograms (1). On the other hand the different species studied gave chromatographic elution profiles exhibiting differences in the relative heights of some of the major peaks. On visual inspection of the chromatograms, *M. avium* (strain NCTC 8551), *M. bovis*, strain BCG, *M. kansasii*, and *M. tuberculosis* could be differentiated (1). Chromatograms reflecting merely the fatty acids of *M. bovis*, strain BCG, and *M. tuberculosis* were similar. However, due to differences in the carbohydrate compositions the TFA technique enabled differentiation even between these two species.

Analyses of mycobacteria belonging to the *M. avium-intracellulare* complex revealed a heterogeneity between the strains studied (11).

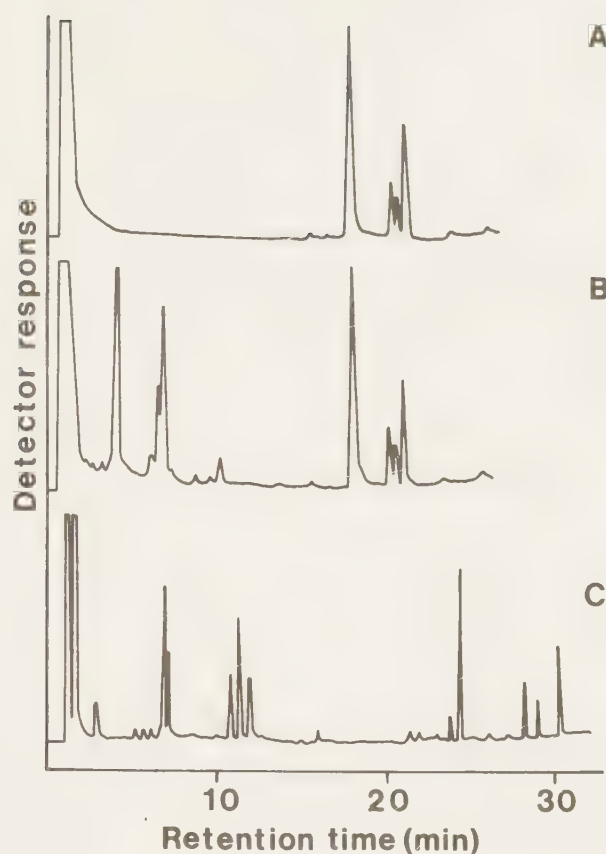


Fig. 2. Chromatograms obtained by analysis of *Mycobacterium tuberculosis*, representing fatty acid methyl esters (A) and products from TFA derivatization of whole-cell methanolysates (B,C). A 2 m packed (A,B) and a 25 m glass capillary (C) column, employing OV-101 as stationary phase, were used.

13 strains were serologically identified as *M. avium* and 9 as *M. intracellulare*. No correlation was found between the TFA chromatographic patterns and the results from the serological tests. Differences were found between chromatograms representing strains within both *M. avium* and *M. intracellulare*, sometimes more obvious than between different species (1). A heterogeneity in the fatty acid composition of these mycobacteria has been reported previously (47). Thus, the GC technique used would appear to provide an alternative means by which to differentiate bacteria belonging to the *M. avium-intracellulare* complex. Whether the organisms within this complex can be differentiated from other mycobacterial species requires further studies.

The value of computerized interpretation of GC elution profiles of microorganisms was demonstrated in study II. The relative areas of six of the major peaks, present in each chromatogram, were used for cluster analysis based on Euclidean distances. The phenogram so obtained enabled a more objective comparison between chromatograms than was possible by mere visual inspection. In subsequent studies, it would therefore seem logical to computerize all the information produced by the integrator and to test also other data analytic methods (21).

The results obtained indicate that analysis by GC of TFA derivatized whole-cell methanolysates is an efficient way of characterizing mycobacteria. Recent studies have revealed that the technique also enables one to distinguish between mycobacteria belonging to Runyon's group II. A glass capillary column was used (unpublished results). In the studies so far, the organisms have been harvested after 28 days of incubation. The amount of cellular material then considerably exceeds that required for GC analysis. It should be evaluated if the incubation time could thus be shortened, meaning that GC would provide a rapid alternative to traditional techniques for the differential diagnosis of mycobacteria. It should also be studied whether mycobacteria can be analysed after being harvested direct from slants of Löwenstein-Jensen medium, which is often employed for the isolation of mycobacteria from clinical specimens.

The GC technique used (TFA) allows simultaneous determination of fatty acids and carbohydrates not only of mycobacteria but also of other microorganisms. The method requires several preparative steps, which need to be rationalized to make it an attractive alternative or complement to other diagnostic procedures in the clinical microbiological laboratory. For this purpose, the possibility of simultaneous extraction and derivatization of several samples, automated injection, and computerized evaluation of the often complex chromatographic patterns obtained, should be evaluated.

DEVELOPMENT OF A GAS CHROMATOGRAPHIC HEAD-SPACE TECHNIQUE FOR THE ANALYSIS OF VOLATILE BACTERIAL PRODUCTS (III,IV)

Background

Analysis of extracellular metabolites is at present the most common application of GC for characterization of clinical isolates of bacteria. Determination by GC of such products has been used in the identification particularly of anaerobic bacteria (54,58, 65,72). Bacterial alcohols and volatile fatty acids are generally chromatographed after being extracted from the broth medium by ethyl ether. Non-volatile fatty acids are converted to volatile derivatives - usually methyl esters - prior to analysis. Volatile compounds can also be analysed by GC by direct injection of the broth medium into the column, preferably after separation of the bacterial cells (73).

Analysis by GC of compounds present in the atmosphere above broth media in a closed bottle, *i.e.* the head-space gas, constitutes another means for distinguishing between microorganisms. Head-space GC (HSGC) was first applied within food and dairy research. Later, the technique was reported to be of value in studies on fungi and bacteria belonging to *Clostridium*, *E. coli* and *Proteus* (59,61-63,70,78). In the light of these results it was therefore decided to try to develop a HSGC technique for determination of the spectrum of volatile metabolites of bacteria. Because of its simplicity, and with its possibilities for automation, HSGC would represent a technique, highly suitable for routine use in the clinical microbiological laboratory.

General theory of HSGC. The area of a chromatographic peak, obtained by HSGC analysis of a compound, *i*, is proportional to its partial vapour pressure at the sampling temperature (90,91), *viz.:*

$$A_i = c_i p_i$$

A_i = peak area,

c_i = constant, depending on the GC detector used,

p_i = partial vapour pressure at the sampling temperature.

The following equation is generally valid:

$$p_i = x_i y_i p_i^0$$

x_i = the mole fraction,

y_i = the activity coefficient,

p_i^0 = vapour pressure of the pure compound at the sampling temperature.

Consequently,

$$A_i = c_i x_i y_i p_i^0.$$

The sensitivity of HSGC, *i.e.* the magnitude of A_i for a given compound, can be improved by obtaining a higher y_i or p_i^0 . The activity coefficient can be markedly changed by adding electrolytes, *i.e.* the "salting out" effect. The vapour pressure can be considerably increased by raising the sampling temperature. The former effect is particularly noticeable for compounds having strong intermolecular electrostatic interactions.

Thus, HSGC may be used in the clinical microbiological laboratory to analyse compounds possessing sufficiently high vapour pressures at temperatures convenient to work with. Compared with the injecting of liquid samples, the technique offers two major advantages. Firstly, when the atmosphere above an aqueous solution is being analysed and a flame ionization detector is used, no solvent peak will appear on the chromatogram, which renders compounds with short retention times detectable. Secondly, since no non-volatile compounds are being introduced into the GC column, its lifespan will be lengthened, as it is protected from deteriorating sample components. Furthermore, the technique is well suited for automation. Units allowing automatic head-space injection are now commercially available (91).

Discussion of results

In studies III and IV, broth cultures were transferred into 10 ml glass vials (Fig. 3). The specimens were then acidified

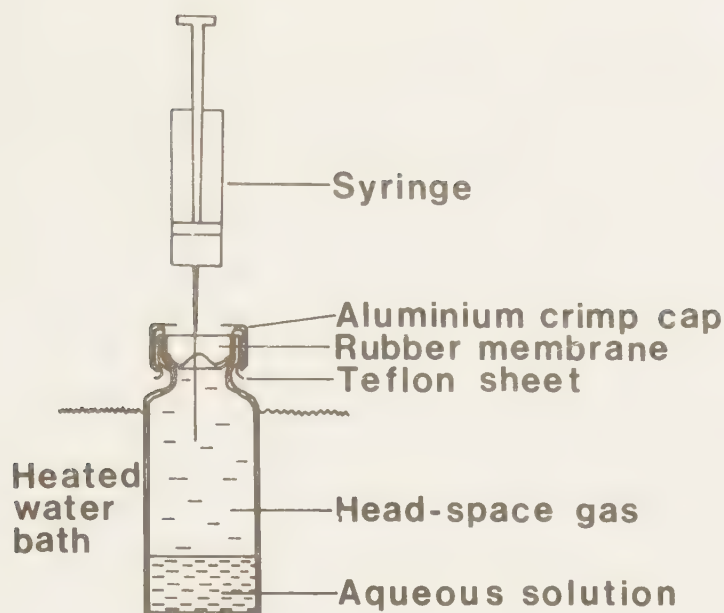


Fig. 3. Glass vial used for gas chromatographic head-space analysis.

or made alkaline, Na_2SO_4 was added, and the vials were sealed using a rubber membrane (protected by a Teflon sheet) and an aluminium crimp cap before being heated in a water bath at 75°C for 10-20 min. Finally, a 2 ml sample of the gas atmosphere was withdrawn with a gas-tight syringe and used for direct injection onto the GC column.

The HSGC technique developed (IV) allows detection of both acidic, neutral and alkaline compounds, provided these can be registered by a flame ionization detector. A single gas chromatograph can be used, equipped with dual columns and detectors, employing Chromosorb 103 for analysis of alkaline and neutral compounds and Porapak Q for analysis of acidic and neutral compounds as stationary phases. The latter phase was found to provide an isothermal separation of alcohols and fatty acids superior to that obtained when using FFAP, and was consequently preferred.

Addition of Na_2SO_4 was found to increase the size of the resulting peaks markedly. The sampling temperature was also found to influence peak heights (III). At 75°C , the largest straight

chain molecules that could be detected contained 8 (for alcohols and fatty acids) and 10 (for amines) carbon atoms, respectively. Temperature equilibrating required 6 min at 75°C, although the peak sizes were not noticeably affected when this period was extended up to several hours. Any absorption of the organic volatiles by the rubber membrane was efficiently hindered by the Teflon sheet.

Under the prevailing working conditions, the peak heights obtained by HSGC analysis of fatty acids containing up to six carbon atoms were comparable to those obtained when injecting broth medium, and extracts of the medium, into the GC column. However, HSGC was superior in sensitivity for the detection of low chain alcohols, thus offering information concerning the production of such metabolites by, for instance, *Clostridium perfringens* and *Propionibacterium acnes*. Peaks corresponding to ethanol, *n*-propanol, and *n*-butanol could be observed only in chromatograms obtained with HSGC. The advantage of HSGC for detecting short-chain alcohols is related to the absence of solvent peaks, hiding early eluting compounds. Furthermore, the high volatility of these compounds consequently leads to their presence in relatively high concentrations in the head-space gas.

Analysis of amines has so far been only rarely employed in GC characterization of microorganisms, and their detection has usually involved rather tedious derivatization procedures using trifluoroacetic or heptafluorobutyric anhydride (55,56,60,67,68,77). However, HSGC was found to offer a very sensitive and easily performed technique for the detection of volatile amines, as exemplified in the studies on *C. septicum* and *Proteus mirabilis* (IV). Comparison between chromatograms obtained through injection into the Chromosorb 103 column of samples, one part of which had been made alkaline and another acidic, showed this procedure to be a tentatively useful method for the identification of amines; peaks present only in the chromatograms produced by alkaline samples represented amines.

Sterile medium was occasionally found to contain relatively large amounts of volatiles, whose chromatographic peaks sometimes

hid early eluting alcohols. This effect, which was more pronounced when using FFAP than Porapak Q as stationary phase, could be reduced by bubbling nitrogen through the medium at 75°C. However, in recent studies we have found that this drawback can be overcome by studying bacterial products from degradation of glucose in a buffer solution. This medium does not contain any organic volatiles. In these studies, bacteria belonging to species most frequently encountered in urinary tract infections were cultured on blood agar plates for 24 h, after which they were transferred to glass vials containing a phosphate buffer and 1 % (w/w) of glucose. After incubation at 37°C for 1 h, head-space GC analysis was carried out, using an automated HSGC analyser (Perkin-Elmer, model F 45). In Fig. 4 a chromatogram of *P. mirabilis*, obtained by this technique, is shown.

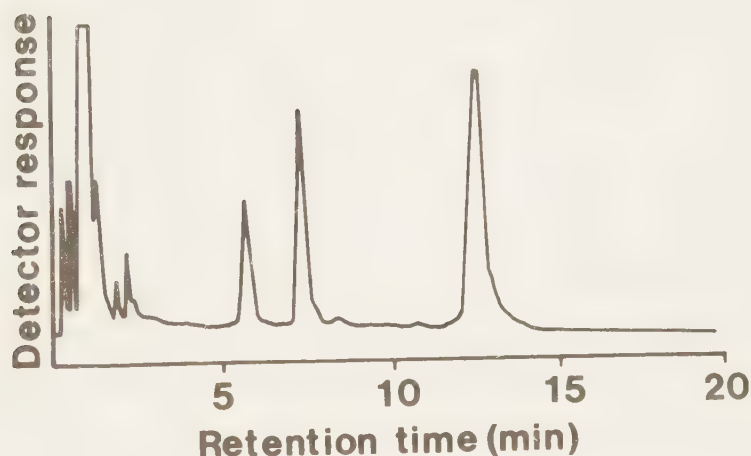


Fig. 4. Analysis by head-space gas chromatography of volatile metabolites of *Proteus mirabilis*, produced by degradation of glucose at 37°C in 1 h.

HSGC can be used for the analysis of any microorganism provided it produces compounds of a high enough volatility. The technique meets several demands for use in the clinical microbiological laboratory. Firstly, the analyses can be automated, thereby avoiding not only the need for staff especially trained in GC, but also

by saving working hours. By means of automated HSGC injector systems (91), analyses can be run on a 24-hour basis. The automation of injections also implies a far better reproducibility than when injecting manually. Secondly, no chemicals, other than salt, a strong acid, and a strong base is needed. Thirdly, HSGC analysis does not require the often laborious preparative steps, *viz.* extraction, centrifugation, derivatization etc which are necessary when employing GC techniques earlier used for analysis of bacterial products.

ANALYSIS OF MYCOCEROSIC AND TUBERCULOSTEARIC ACID USING SELECTED ION MONITORING (SIM) (V,VI)

Background

GC/MS - SIM can be employed for the registration of organic compounds present in biological specimens. The technique has been used, *inter alia*, for diagnosis of inborn errors of the metabolism in humans by the selective and sensitive detection of metabolites (92,93). It therefore seemed likely that SIM would also constitute a possibility for the detection of metabolic products of micro-organisms, both in cultures and directly in clinical specimens.

GC as a tool for the rapid diagnosis of infectious diseases by analysis of various body fluids, such as cerebrospinal and synovial fluids, pleural effusions, pus, serum and urine specimens for alcohols, amines, carbohydrates, and fatty acids has been tested previously (55,74-84). Some of the resulting chromatographic patterns have been reported as being specific for given infectious diseases. However, chromatograms obtained in analyses of specimens from patients suffering from infections of the same etiology have not always been identical. Diagnostic problems may also arise in cases of mixed infections.

If metabolites unique for a given microbial genus or species could be demonstrated, these two obstacles would be eliminated. It was therefore decided to determine whether SIM could be applied in detecting such products in body fluids and, for comparison, in bacterial cultures too (V,VI). It was decided to study mycobacterial cells and sputum samples from patients with pulmonary

tuberculosis, because of the time-consuming and tedious procedures required for diagnosis of tuberculosis (*cf.* page 10).

Mycobacteria contain relatively large quantities of fatty acids, which have been studied extensively over several decades. The mycocerosic acids constitute a group of polymethyl-substituted, optically active acids of high molecular weight. They have been found exclusively in the lipids of *M. bovis* and *M. tuberculosis* (94,95). Tuberculostearic acid, being monomethyl-substituted, is also optically active and has been demonstrated not only in a number of mycobacterial species, but also in some species of *Nocardia* and *Streptomyces* (96). The mass spectrum of the methyl ester of tuberculostearic acid (10-methyloctadecanoic acid) exhibits a number of prominent peaks in the high mass range (Fig. 5). Fragments of m/z 167, 171, and 199 are all related to cleavage at the methyl chain (position 10), while the molecular peak is found at m/z 312. The spectrum representing the methyl ester of C32 mycocerosic acid (2,4,6,8-tetramethyloctacosanoic acid) is dominated, in the high mass range, by the molecular peak at m/z 494 (4).

Discussion of results

In the present investigation, tuberculostearic and C32 mycocerosic acid were selected for study by SIM analysis of both mycobacterial cultures (V) and sputum specimens from patients with pulmonary tuberculosis (VI). The acids were analysed as methyl esters. Ions of m/z 167 and 312 were used for the detection of tuberculostearic acid, while the spectrometer was focused at m/z 494 when studying C32 mycocerosic acid.

The sensitivity for detection of C32 mycocerosic acid was considerably weaker than that for tuberculostearic acid. The detection limit of the latter was 20 pg (monitoring at m/z 312). Methyl tuberculostearate could be detected with a greater sensitivity when monitoring at m/z 312 than at m/z 167. This can be explained by differences in the relative intensities of corresponding ions. Thus, in Fig. 5 it can be observed that the peak of m/z 312 is higher than that of m/z 167.

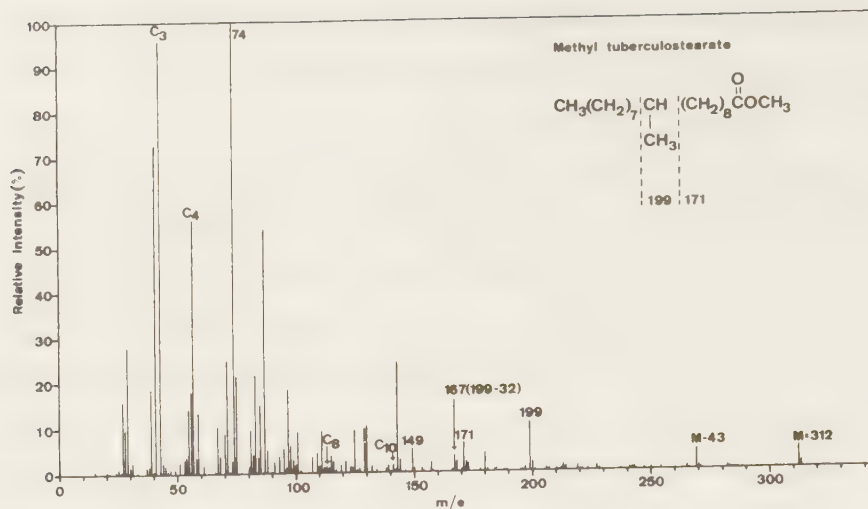


Fig. 5. Electron impact mass spectrum of natural methyl tuberculostearate.

Analysis using SIM (monitoring at m/z 167 or m/z 312) of methanolysed lipid extracts of mycobacterial cells gave chromatograms containing only one peak, *viz.* that of methyl tuberculostearate (Fig. 6A). Corresponding analyses of a great number of microorganisms not belonging to *Mycobacterium*, *Nocardia* or *Streptomyces* resulted in totally blank chromatograms. These organisms included a number of bacteria known to be capable of causing pneumonia (V). When analysing sputum specimens at m/z 312, that had been found to contain acid-fast rods by staining, several other components were also registered (Fig. 6B). These additional peaks were identified by GC retention times as the methyl esters of 2- (a) and 17- (c) methyloctadecanoic acid, and of nonadecanoic acid (d). Peak (b) corresponds to methyl tuberculostearate. Tuberculostearic acid could also be demonstrated when monitoring at m/z 167, although the chromatographic patterns obtained were more complicated (VI).

The presence of tuberculostearic acid in cultures of *M. tuberculosis* on Löwenstein-Jensen slants could readily be established 5 days after inoculation, that is, weeks before colonies could be observed with the naked eye (Fig. 6A). This fatty acid could also be detected in sputum specimens of patients with pulmonary

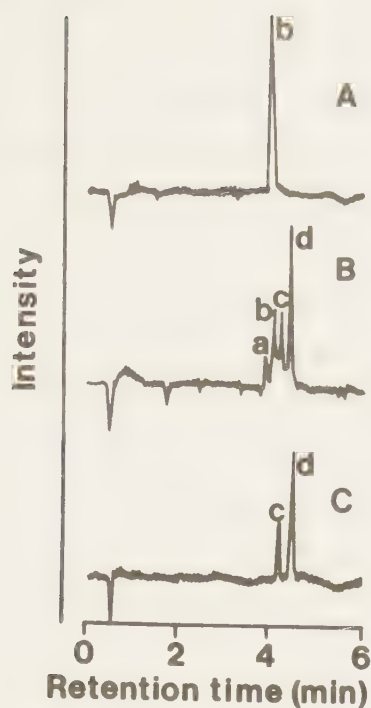


Fig. 6. SIM analysis monitoring at m/z 312 of a 5-day culture of *M. tuberculosis* (A), a sputum specimen obtained from a patient with pulmonary tuberculosis (B) and from another patient with non-tuberculous pneumonia (C). Lipid extracts of the samples were subjected to reaction with methanol under acidic conditions, extraction by *n*-hexane, and purification using thin-layer chromatography.

tuberculosis (Fig. 6B). Its detection was made possible thanks to the high selectivity and sensitivity of SIM in detecting methyl tuberculostearate. However, preliminary unpublished results have revealed that even better results might be achieved by using chemical ionization which offers at least a 5-fold increase in sensitivity. In addition, alternative derivatives can be used. For example, *tert*-butyldimethylsilyl derivatized tuberculostearic acid, analysed by electron impact SIM at m/z 355, improves sensitivity by a factor of approximately 5 compared with studying the methyl ester when focusing at m/z 312 (unpublished results).

More extensive investigations to assess the usefulness of the SIM technique for diagnosis of mycobacterial infections in the clinical microbiological laboratory are now in progress. These studies will also include analyses of biopsy, gastric lavage, and pleural effusion specimens from patients with tuberculosis. Tuberculostearic acid might be present in such specimens in too small amounts to be identified by MS. Its identity can therefore only be established by specifying GC retention data. Thus, it is important that the chromatographic conditions used provide an efficient separation. Capillary columns are preferred.

As exemplified in the present study of tuberculostearic acid, SIM is an extremely sensitive technique for the detection of bacterial products. It requires little laborious preparative work thanks to the selectivity of the registration. Furthermore, automatic injection units can now be coupled to GC/MS instruments. Many microbial genera or species are known to produce unusual or possibly unique metabolites, why SIM is likely to become an established diagnostic tool in clinical microbiology.

GENERAL SUMMARY

Gas chromatography (GC) and mass spectrometry (MS) were used for the registration and identification of bacterial products. Both cellular and extracellular metabolites were studied, *e.g.* carbohydrates, fatty acids, alcohols and amines. In addition, selected ion monitoring (SIM) was applied for the quantitative determination of bacterial fatty acids.

Carbohydrate and lipid constituents of mycobacteria were simultaneously analysed by GC as trifluoroacetyl derivatized methyl glycosides and fatty acid methyl esters, respectively. Each of the mycobacterial strains studied could be characterized by its (highly reproducible) chromatographic pattern. Thus, the GC technique used appears to be a valuable aid in the differential diagnosis of mycobacteria. It also offers the possibility to a more rapid identification of mycobacteria than is allowed by the use of traditional diagnostic methods.

A GC head-space technique for the analysis of volatile microbial products has been developed and applied for bacterial characterization. The method allows registration of alkaline, neutral and acidic compounds and provides a far better detection of, *inter alia*, short-chain alcohols and amines than the earlier GC techniques used for analysis of bacteria. By eliminating any need for extraction and derivatization procedures, GC head-space analysis is well suited for automation and constitutes a convenient diagnostic procedure in the clinical microbiological laboratory.

The adaptation of GC/MS for SIM made quantitative determinations of bacterial fatty acids possible, even when present as traces of only a few picograms. By the registration of tuberculostearic acid, the presence of *Mycobacterium tuberculosis* inoculated onto agar slants could be established weeks before colonies could be visually observed. In addition, tuberculostearic acid was demonstrated in sputum specimens of patients with pulmonary tuberculosis. SIM would therefore seem to be a useful technique for the rapid diagnosis of tuberculosis and other mycobacterial infections.

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